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13. ABSTRACT *(Maximum 200 Words)*

The HER-2/neu extracellular domain (ECD) is shed from breast carcinoma cells in culture and is found at elevated levels in sera of patients with metastatic breast cancer where it may predict poor prognosis. Our studies show that an N-terminally truncated HER-2/neu product, p95, is produced when the ECD is shed, that p95 has kinase activity, and is expressed to a greater extent in breast cancer patients with lymph node metastasis. **Purpose.** The objective of this proposal is to directly test the hypothesis that shedding of the extracellular domain of HER-2/neu, which creates the truncated p95 kinase, promotes oncogenesis. **Scope.** The effect of shedding to oncogenesis will be examined by further characterizing the control of shedding and genetically altering shedding activity to test the impact on tumorigenesis and oncogenesis. **Results:** We have developed and characterized strategies for generation of mutants of p185HER-2 to alter shedding. We have characterized the activity of mutants with altered juxtamembrane sequences. We have been unsuccessful in development of mutants that specifically alter shedding, but that do not affect other receptor activities. These mutant proteins either are unstable or have altered kinase activity. Other recent studies lend further support to the concept that the region of the ectodomain, adjacent to the transmembrane region is critical in the regulation of dimerization and kinase activation. Therefore juxtamembrane mutants are considered problematic for examining the function of shedding on receptor-mediated tumorigenesis. We now propose to introduce additional approaches that employ chemical inhibitors and activators of shedding to examine the impact on transformation and tumorigenesis.

14. SUBJECT TERMS **HER-2/neu (erbB-2), receptor tyrosine kinases, proteolytic shedding of membrane proteins, *in vitro* transformation assays, tumorigenesis and metastasis assays.**

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INTRODUCTION

Subject: The HER-2/neu extracellular domain (ECD) is shed from breast carcinoma cells in culture and is found at elevated levels in sera of patients with metastatic breast cancer where it may predict poor prognosis, response to adjuvant endocrine and chemotherapy, and allow tumor cells to escape immune surveillance. Our studies show that an N-terminally truncated HER-2/neu product, p95, is produced when the ECD is shed, has kinase activity, and is expressed to a greater extent in breast cancer patients with lymph node metastasis (1). **Purpose:** The objective of this proposal is to directly test the hypothesis that shedding of the extracellular domain of HER-2/neu and creating of the truncated p95 kinase promotes oncogenesis. **Scope:** To examine the impact of shedding on oncogenesis: (a) We will genetically alter shedding of HER-2/neu. To alter shedding, deletion and domain replacement mutants will be constructed within the HER-2/neu juxtamembrane cleavage domain. The mutations to be made will be based on known structural determinants of shedding defined through studies of diverse transmembrane proteins. A second approach to genetically alter shedding will be pursued by expressing HER-2/neu in cells that are null for shedding enzyme. (b) The impact of altered shedding to oncogenesis will be examined in cell culture and animal models. The transforming activity of HER-2/neu with genetically altered levels of shedding will be examined by well-established cell culture models of transformation, by tumorigenesis assays in nude mice, and by metastatic potential in immune compromised mice.

BODY

STATEMENT OF WORK

The following outlines the statement of work to be conducted and the progress we have made in this direction.

Task 1. Genetically alter the proteolytic shedding of p185HER-2/neu (months 1-24).

To examine the impact of shedding of the ectodomain of p185HER-2 on tumorigenesis, we proposed to alter the extent of shedding by engineering altered sequence in the juxtamembrane domain of p185HER-2. The juxtamembrane region of the ectodomain is known to be the site of proteolytic cleavage of transmembrane proteins

during shedding (Arribas et al., 1996). The mutants were then to be characterized first for their proper location at the cell surface and for kinase activity by examining autophosphorylation of the mutant receptor and by analyzing the kinase enzymatic activity of the receptor by immunoprecipitation and in vitro kinase activity. The characterization of the mutant receptor was an important first step since alterations in biosynthesis, processing, or kinase activity would be expected to affect tumorigenic potency of the receptor obscuring effects of shedding.

The first mutant to be constructed was a deletion of the juxtamembrane stalk of p185HER-2 and the second was an altered sequence of the juxtamembrane stalk created by swapping with the juxtamembrane of tumor necrosis factor-α precursor (preTNFα), which undergoes potent tumor promoter inducible shedding. We used PCR mutagenesis to delete the 12 residue stalk of HER-2/neu, which is bordered by the conserved Cys at position 642 and the transmembrane domain beginning at Ile 654. The PCR product, cloned into the pcDNA 3.1 mammalian expression plasmid, was then sequenced. Initial analysis of the sequence in the first year of funding suggested introduction of an artifactual point mutation that introduced a stop codon in the region that encodes the extracellular domain of p185HER-2 presumably creating a truncated protein. We altered PCR conditions and have managed to generate a juxtamembrane deletion mutant in a pcDNA3.1 expression plasmid. Nucleotide sequencing of the mutant demonstrated that the juxtamembrane regions was deleted with no other detectable mutations. The initial characterization of the mutant involved transient transfections of Cos-7 cells and analysis of mutant protein production by Western blot analysis. The wildtype HER-2 in the same expression plasmid, pcDNA3.1, was used as a positive control for transfection efficiency and for production levels of p185HER-2 protein. While the wildtype protein, p185HER-2 was produced in abundant amounts, we were unable to detect production of the juxtamembrane deletion mutant. When large amounts of transiently transfected cells were subjected to immunoprecipitation followed by Western blot analysis to detect signal, we occasionally detected small amounts of the protein that could have been the mutant based on size and antibody reactivity. We next attempted to select stably transfected cells (3T3) cells using G418 selection. Clonal populations were selected and analyzed for production of juxtamembrane deletion mutant of p185HER-2. While several colonies were analyzed, the mutant HER-2 protein could not be detected. It was concluded that deletion of the juxtamembrane of p185HER-2 protein resulted in folding defects leading to deficient protein production.

We next designed a strategy to delete the 16 amino acids adjacent to the transmembrane domain, to exactly mimic an alternative splicing event described by Siegel et al., (1999), which results in deletion of an exon. The removal of the exon in this alternatively spliced HER-2 form caused a deletion of 16 amino acid residues adjacent to the transmembrane domain, which effectively removed the stem sequence. We chose to analyze this deletion mutant since it mimics the naturally occuring splice variant and therefore would be expected to be properly folded and efficiently produced. However, authors of this paper reported that this splicing variant has enhanced transforming activity. Further biochemical characterization of this variant product of HER-2 has revealed that the protein has enhanced in vitro kinase activity. Altered kinase activity and enhanced transforming activity of this mutant protein precludes efforts to examine

effects of altered shedding on tumorigenesis, since any effect of shedding would be secondary to altered kinase activity.

Another plan for generating a mutant with altered shedding activity was to swap the juxtamembrane domain of tumor necrosis factor-α precursor (preTNFα) with that of p185HER-2. The shedding of TNFα is efficiently induced by addition of tumor promoters (Blobel, 1997). In contrast p185HER-2 shedding is very slow and is not inducible by tumor promoters (Christianson et al., 1998, Codony-Servat et al., 1999). Replacement of the p185 juxtamembrane domain with that of TNFα should create a p185HER-2 mutant with greatly enhanced inducible shedding. To create the chimeric receptor, we digested the p185HER-2 cDNA with AatII, which cuts at a unique site immediately adjacent to the p185HER-2 transmembrane coding sequence. We then ordered an oligonucleotide that is identical to the 36 nt sequence encoding the TNF-α stem sequence that also contains an AatII restriction site at each end. The oligonucleotide was then ligated into the AatII digested p185HER-2 cDNA. We followed the exact procedure of Codony-Servat et al., (1998) who also constructed a swap of the juxtamembrane domain of p185HER-2 for that of proTGFα, to modulate the shedding of p185HER-2. They showed that the p185HER-2 mutant indeed had tumor promoter induced shedding at a much greater rate than the wildtype p185HER-2. Although Codony-Servat et al., (1999) showed that tumor promoter induced shedding of this mutant, they did not characterize its kinase activity or other properties. To examine the properties of this mutant, we again performed transient transfection into Cos-7 cells. The protein was produced and detected by immunoblotting. To further characterize this mutant, we examine the kinase activity by examining the tyrosine phosphorylation level by conducting anti-phosphotyrosine blots. In this system, overexpression of the wildtype p185HER-2 results in dimerization and constitutive tyrosine phosphorylation. Comparisons of the tyrosine phosphorylation level of the mutant and wildtype have initially revealed that the mutant has decreased levels suggested that the kinase activity is altered. In the next funding period we will further characterize the properties of this mutant including its biosynthesis, cell surface expression level, tyrosine kinase activity, and extent of tumor promoter induced shedding compared to the wildtype receptor. These initial characterizations will be used to determine whether this mutant will be of use in sorting out the effects of altered shedding versus the direct effects of the mutation on the receptor activity. Another drawback to the use of this mutant is that induction of shedding requires treatment with tumor promoters, which are known to alter the growth properties and the tumorigenic activity. Therefore interpretation of the effects of shedding will be complicated by the effects of the tumor promoters that are required to induce shedding.

In summary, several findings have caused us to adopt new approaches in addition to construction of mutations in the juxtamembrane region of p185HER-2 to alter shedding. Alternative strategies will be required since we were unable to express our juxtamembrane deletion mutant possibly due to improper folding. Secondly, the 16 amino acid deletion of the juxtamembrane region found in the recently described splice variant of HER-2 has been found to increase transforming activity and kinase activity. Therefore any effects on shedding would be secondary to this activity. Third, the domain swapping mutant of TNF-α stem sequence for the p185HER-2 juxtamembrane region appears to alter receptor tyrosine phosphorylation levels, and importantly, the tumor

promoters (phorbol esters) required to induce shedding of the TNF-α precursor affect cell growth properties and tumorigenesis. The problems involved in interpretation of results obtained using juxtamembrane mutations are further emphasized by recent studies (Burke and Stern, 1998) showing the importance of the juxtamembrane domain sequence of p185HER-2 in dimerization and receptor activation. This study suggests that any attempts to disrupt the juxtamembrane region will have the primary consequence of affecting receptor activation.

In the final year of funding we proposed to incorporate alternative methods to alter shedding in order to test the impact of shedding on the tumorigenic activity of p185HER-2. The statement of work and the overall goals will remain the same. However instead of the sole reliance of juxtamembrane mutants for altering shedding, we will also alter shedding thought the use of cleavage inhibitors and activators that will be used alone and in combination. For inhibitors, we will use TAPI (Christianson et al., 1998) and BB-94 (Codony-Servat et al., 1999) both metalloprotease inhibitors shown to inhibit HER-2 shedding. To stimulate shedding, we will use the 4-aminophenylmercuric acetate (APMA), a well-known matrix metalloprotease activator known to cause potent stimulation of HER-2 shedding (Molina et al., 2001). Initially HER-2 transfected 3T3 cells and control 3T3 cells will be treated with the inhibitors or the metalloprotease activator APMA to alter shedding. The control cells and HER-2 overexpressing cells will then be examined for focus formation and anchorage independent growth. It will be important to use the control 3T3 cells to ascertain effects of these activators and inhibitors that are not specifically due to altered HER-2 shedding. For a cell model that is more relevant to breast cancer, we will also conduct the same type of study on MCF-7 and HER-2 transfected MCF-7 breast carcinoma cells. If we can determine effects that are specific for HER-2, then tumorigenesis in nude mice will be examined. It may be necessary to first assess the toxicity of any of these compounds in animal before testing the tumorigenesis capacity of the different cell lines.

KEY RESEARCH ACCOMPLISHMENTS

- Successfully constructed a juxtamembrane deletion mutant.
- Characterized the juxtamembrane deletion mutant by transient and stable transfections of cells. Determined a defect in protein production suggesting problems with folding of the mutant protein.
- Obtained a p185HER-2 mutant with the juxtamembrane domain swapped for that of the TNF-α precursor protein. Further characterization of this mutant revealed altered kinase activity. Moreover, shedding requires treatment with tumor promoters, which alter tumorigenic activity of transfected cells.
- Have determined that TACE is not likely the proteolytic enzyme involved in shedding of p185HER-2. Similar p185HER-2 shedding activity was observed in TACE null versus wildtype cells stably transfected with HER-2.
- Have determined that the defect in shedding from the SKOV-3 cells is not due to altered p185HER-2 structure and therefore may be caused by reduced levels of shedding enzyme.

- Have not been able to detect the alternative spliced product of p185HER-2 with the altered juxtamembrane domain in two carcinoma cells lines.

REPORTABLE OUTCOMES

- Manuscripts, abstracts, presentations:
  None.

- Patents and licenses applied for and/or issued:
  None
- Degrees obtained that are supported by this award:
  None
- Development of cell lines, tissue, or serum repositories:
  None
- Informatics such as databases and animal models:
  None
- Funding applied for based on work supported by this award:
  None
- Employment or research opportunities applied for and/or received :
  None

CONCLUSIONS

To summarize, we have developed strategies for constructing deletions mutants and domain substitution mutants to alter the extent of shedding. Because of altered receptor processing and kinase activity caused by these mutations and recent information suggesting that the juxtamembrane region of p185HER-2 is critical for receptor dimerization and kinase activity, we have decided that generation of juxtamembrane mutants will not be an effective approach for testing the role of shedding of the ectodomain in receptor mediated tumorigenesis. While the statement of work remains the same, we propose to use alternative approaches involving the use of chemical inhibitors and stimulators of shedding described above for testing the effects of shedding. The hypothesis that TACE may be the enzyme involved in shedding of HER-2 and the plans to use TACE null cells to control shedding have not worked. Our studies suggest that a shedding enzyme, rather than TACE may be involved in shedding of p185HER-2.

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APPENDICES

None